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Functional characterization of three genes encoding putative oxidoreductases required for cercosporin toxin biosynthesis in the fungus *Cercospora nicotianae*

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Cercosporin is a non-host-selective, photoactivated polyketide toxin produced by many phytopathogenic *Cercospora* species, which plays a crucial role during pathogenesis on host plants. Upon illumination, cercosporin converts oxygen molecules to toxic superoxide and singlet oxygen that damage various cellular components and induce lipid peroxidation and electrolyte leakage. Three genes (*CTB5*, *CTB6* and *CTB7*) encoding putative FAD/FMN- or NADPH-dependent oxidoreductases in the cercosporin toxin biosynthetic pathway of *C. nicotianae* were functionally analysed. Replacement of each gene via double recombination was utilized to create null mutant strains that were completely impaired in cercosporin production as a consequence of specific interruption at the *CTB5*, *CTB6* or *CTB7* locus. Expression of *CTB1*, *CTB5*, *CTB6*, *CTB7* and *CTB8* was drastically reduced or nearly abolished when *CTB5*, *CTB6* or *CTB7* was disrupted. Production of cercosporin was revived when a functional gene cassette was introduced into the respective mutants. All *ctb5*, *ctb6* and *ctb7* null mutants retained wild-type levels of resistance against toxicity of cercosporin or singlet-oxygen-generating compounds, indicating that none of the genes plays a role in self-protection.

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INTRODUCTION

Many phytopathogenic fungi are equipped with various arsenals such as secretion of cell-wall-degradation enzymes and formation of phytotoxins in order to invade their hosts (Schafer, 1994). Host-selective toxins kill plant cells by targeting a specific cellular enzyme or component, and thus are toxic only to a limited range of host cultivars (Walton, 1996). In contrast, non-host-selective toxins, targeting various cellular components, enable the producing pathogens to have wide host ranges. Cercosporin (Fig. 1a) is a non-host-selective perylenequinone toxin produced by many phytopathogenic *Cercospora* species, which have been reported to affect several hundred plant species, including many major crops such as corn, rice, banana, coffee, sugar beet, soybean, peanut and tobacco (reviewed by Daub & Ehrenshaft, 2000; Daub *et al.*, 2005). Cercosporin is an important virulence determinant in *Cercospora* species (Callahan *et al.*, 1999; Choquer *et al.*, 2005, 2007; Dekkers *et al.*, 2007; Shim & Dunkle, 2003; Upchurch *et al., 1991*). Compared to other non-host-selective phytotoxins, cercosporin has several unique features, including light activation for its biosynthesis (Ehrenshaft & Upchurch, 1991), light- and oxygen-dependent cytotoxicity (Yamazaki *et al.*, 1975), and the production of reactive oxygen species such as singlet oxygen and superoxide (Daub & Hangarter, 1983). In the past two decades, intensive research has been focused on elucidation of the genetic mechanisms involved in self-protection from cercosporin and singlet-oxygen-generating compounds (Chung *et al.*, 1999, 2003a; Daub *et al.*, 1992, 2000; Ehrenshaft *et al.*, 1998, 1999; Jenns & Daub, 1995; Sollod *et al.*, 1992).

Cercosporin was first isolated from the soybean pathogen *Cercospora kikuchii* in 1957 (Kuyama & Tamura, 1957) and its structure was chemically determined by Yamazaki & Ogawa (1972). In addition to plants, cercosporin has been

Abbreviations: CTB, cercosporin toxin biosynthesis; CSPD, disodium 3-[4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl]phenyl phosphate; dUTP, 2′-deoxyuracil 5′-triphosphate; FGSC, Fungal Genetics Stock Center; PDA, potato dextrose agar.

The GenBank/EMBL/DDBJ accession numbers. for the *CTB5*, *CTB6* and *CTB7* sequences of *Cercospora nicotianae* are DQ991507, DQ991508 and DQ991509 respectively.

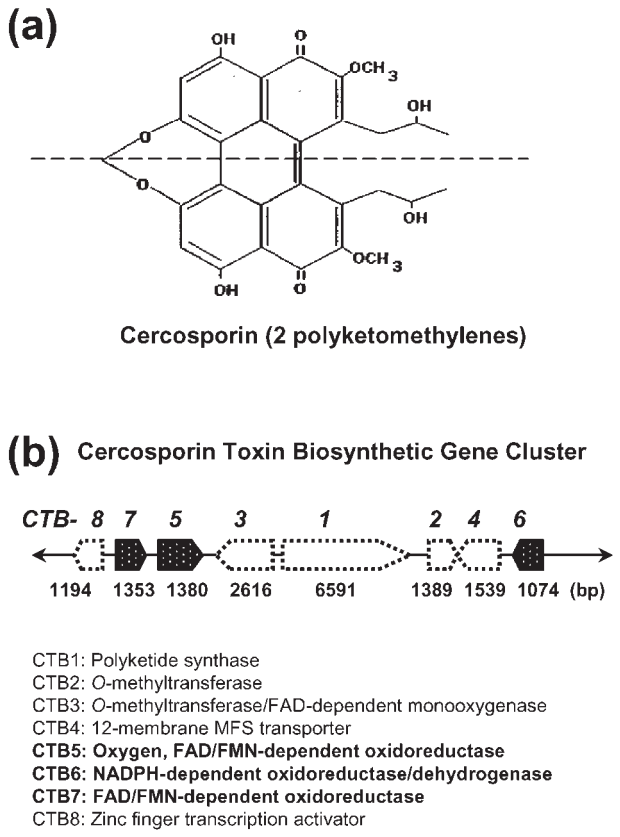

Fig. 1. Cercosporin toxin produced by *Cercospora* spp. and the biosynthetic *CTB* gene cluster: (a) Chemical structure of cercosporin [1,12-bis-(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone], containing two identical polyketomethylene units (separated by the dashed line). (b) Schematic illustration of the cercosporin toxin biosynthetic gene cluster (*CTB1–8*) with predicted functions in *C. nicotianae*. Arrows indicate the orientation of transcription. Sizes of the coding sequences are also indicated.

shown to be toxic to various cell types, including bacteria and many fungi and animal cells, due to the production of reactive oxygen species (Daub *et al.*, 2005). Cercosporin is capable of breaking down different cellular components such as lipids, proteins and nucleic acids, depending on its localization in cells. During pathogenesis in host plants, cercosporin has been shown to damage cell membranes by causing lipid peroxidation, electrolyte leakage and eventually cell death (Daub, 1982; Daub & Briggs, 1983).

Based on substrate feeding experiments (Okubo *et al.*, 1975) cercosporin has been proposed to be synthesized by a fungal polyketide pathway. However, the genes or enzymes involved in cercosporin production remain largely unknown. Recent studies with molecular and genetic tools began to uncover the cercosporin biosynthetic pathway and regulation network (Daub *et al.*, 2005). Light has long been known to be required for cercosporin toxicity. Biosynthesis of cercosporin is also primarily triggered by light (Jenns et al., 1989). In order to elucidate cercosporin biosynthesis and regulation at molecular levels, several cercosporin-deficient mutants were identified via a restriction-enzyme-mediated mutagenesis approach (Chung *et al.*, 2003b). As a result, two linked genes, *CTB1* (encoding a polyketide synthase) and *CTB3* (encoding a dual methyltransferase/monooxygenase) that were required for cercosporin biosynthesis were identified and characterized from *Cercospora nicotianae* (Choquer *et al.*, 2005; Dekkers *et al.*, 2007). We later obtained nine additional ORFs beyond the boundaries of *CTB1* and *CTB3* by combining chromosome walking and sequence analysis. Six of them (*CTB2*, *CTB4*, *CTB5*, *CTB6*, *CTB7* and *CTB8*) encode polypeptides proposed to be involved in cercosporin production (Chen *et al.*, 2007). Expression of eight of the genes was induced under cercosporin-producing conditions and was co-ordinately regulated by the $Zn(II)Cys_6$ transcriptional activator, CTB8 (Chen *et al.*, 2007). We hypothesize that cercosporin, like many fungal secondary metabolites (Keller *et al.*, 2005), is synthesized by a cluster of co-regulated genes, in this case designated the cercosporin toxin biosynthesis (*CTB*) gene cluster. The functions of *CTB1*, *CTB2*, *CTB3*, *CTB4* and *CTB8* in cercosporin biosynthesis have been unambiguously demonstrated by analysing the respective null mutants (Chen *et al.*, 2007; Choquer *et al.*, 2005, 2007; Dekkers *et al.*, 2007). The functions of *CTB5*, *CTB6* and *CTB7*, which encode proteins similar to numerous FAD/FMN- or NADPH-dependent oxidoreductases or dehydrogenases, remain to be elucidated. In the present study, we characterized these genes by creating loss- and gain-of-function strains; we present conclusive evidence to demonstrate their roles in cercosporin biosynthesis, and completely define the core *CTB* gene cluster.

METHODS

Fungal isolates and culture conditions. Wild-type *C. nicotianae* (ATCC 18366) and genetically modified strains were maintained on complete medium (CM) (Jenns *et al.*, 1989). Cercosporin-production mutants were screened daily for the lack of red pigment production on thin potato dextrose (glucose) agar (PDA, Difco, Becton, Dickinson and Company) plates by a method described previously (Chung *et al.*, 2003b). We found that a thin PDA plate (less than 15 ml medium in a 100×15 mm Petri dish) supported the highest production of cercosporin under illumination. Assays for sensitivity to photosensitizing compounds (cercosporin, eosin Y, haematoporphyrin, methylene blue or toluidine blue) were performed by growing fungal isolates on CM containing 10 or 100 μM test compound under continuous light as described previously (Jenns & Daub, 1995). All chemicals were purchased from Sigma-Aldrich unless otherwise stated, and dissolved appropriately in acetone or water to make a 10 or 100 mM stock solution.

Cercosporin analysis. Cercosporin was extracted with 5 M KOH or with ethyl acetate from agar plugs with mycelia as described previously (Choquer *et al.*, 2005; Chung, 2003). Cercosporin in KOH extracts was quantified by measuring A_{480} using a Genesys 5 spectrophotometer (Spectronic Instruments). Ethyl acetate extracts were analysed on a TLC plate coated with a 60 F254 fluorescent silica

gel with ethyl acetate/hexane/methanol/H_2O (6 : 4 : 1.5 : 1, by vol.) as a solvent (Choquer *et al.*, 2005).

***CTB5*, *CTB6* and *CTB7* gene disruption.** A strategy employing the split-hygromycin phosphotransferase B gene (*HYG*) marker fused with truncated *CTB5*, *CTB6* or *CTB7* fragments was used for targeted gene disruption in *C. nicotianae* as described previously (Choquer *et al.*, 2005). All DNA clones were built on the backbone of pGEM-T easy vector (Promega). For *CTB5* disruption, a 3.3 kb fragment was amplified with primers CTB5R (5′-GCTACAGTGCGACGGAGT-CCTG-3′) and CTB7I (5′-CTCGGCCGCGAGAAGGCTT-3′) from genomic DNA and cloned to yield pCTB5. A 1.0 kb *Hin*dIII–*Age*I fragment in pCTB5 was replaced with a 1.6 kb *Bam*HI, end-filled fragment harbouring the *HYG* cassette from pUCATPH [obtained from the Fungal Genetics Stock Center (FGSC), University of Missouri, Kansas City, MO, USA] to generate the disruption construct, pctb5. Two truncated *HYG* and *CTB5* fusion fragments overlapping within the *HYG* region (800 bp) were amplified by PCR from pctb5, and directly transformed into wild-type *C. nicotianae* for gene disruption. A 2.3 kb fragment containing 5′ *CTB5* fused with 3′ *HYG* was amplified with primers CTB7I and Hygsplit2 (5′-CCG-ACAGTCCCGGCTCCGGATCGG-3′); a 2.1 kb fragment containing 5′ *HYG* fused with 3′ *CTB5* was amplified with primers CTB5R and Hygsplit1 (5′-AGGAGGGCGTGGATATGTCCTGCGGG-3′).

For *CTB6* disruption, a 2 kb fragment was amplified with primers ctb6F (5′-CAAACGCAGATACCTCGCCGCATG-3′) and mfs4 (5′-GCAAATTCTGAGGATTTCCCTTG-3′), and cloned to form pCTB6. A 0.5 kb *Bgl*II–*Hin*dIII fragment in pCTB6 was replaced with the *HYG* cassette to generate pΔ*ctb6*. Two fragments, of 1.7 and 1.9 kb, overlapping within *HYG*, were obtained from pΔ*ctb6* with primers ctb6F and hygsplit1 and mfs4 and hygsplit2, respectively.

For *CTB7* disruption, a 5 kb fragment was amplified with primers tf4 (5′-CCATGAAGCGAGATGC-3′) and ord3 (5′-CGTATACCGCTA-CCCATGTCGTAC-3′), and cloned to become pCTB7. A 0.9 kb *Eco*47III fragment was replaced with the *HYG* cassette to yield the disruption construct, pΔ*ctb7*. Split-*HYG* marker fragments, of 2.5 and 1.5 kb, were amplified from pΔctb7 with primers tf4 and hygsplit1 and ord3 and hygsplit2, respectively.

Complementation and fungal transformation. The full-length *CTB5*, *CTB6* or *CTB7* ORF, including the corresponding endogenous promoter, was independently amplified with gene-specific primers by a high-fidelity DNA polymerase (Roche Applied Science). Genetic complementation was performed by co-transformation of a PCR fragment with the pCB1532 plasmid carrying the *Magnaporthe grisea* acetolactate synthase gene (*SUR*) cassette for sulfonylurea resistance (Sweigard *et al.*, 1997, obtained from FGSC) into Δ*ctb5*-D8, Δ*ctb6*-D18 or Δ*ctb7*-D2 null mutants. Transformants were selected against sulfonylurea and screened for cercosporin production. Fungal protoplasts were prepared and transformed using $CaCl_2$ and PEG by methods described previously (Chung *et al.*, 2002). Transformants were selected on medium containing 250 μg hygromycin ml^{-1} (Roche), or 5 μg chlorimuron ethyl ml^{-1} (Chem Service) as appropriate and tested for cercosporin production on PDA plates (Chung, 2003).

Sequence analysis. Fungal DNA was isolated with a DNeasy Plant Mini kit (Qiagen). Full-length *CTB5*, *CTB6* or *CTB7* was amplified with the respective primers as described above and cloned into pGEM-T easy vector (Promega) for sequence analysis from both directions at Eton Bioscience. PCR primers were synthesized by Integrated DNA Technologies. Searches for sequence similarity and functional domains were performed with web-based software programs as described previously (Chen *et al.*, 2007). Sequence data from this study have been deposited with the EMBL/GenBank Data Libraries under accession nos. DQ991507 (*CTB5*), DQ991508 (*CTB6*) and DQ991509 (*CTB7*).

Molecular techniques. Standard procedures were used for endonuclease digestion of DNA, electrophoresis, and Southern and Northern blot hybridization. The hybridization probes were generated by PCR with gene-specific primers to incorporate DIG-11-dUTPs (Roche) into *CTB1*, *CTB5*, *CTB6*, *CTB7* or *CTB8* DNA fragments as previously described (Choquer *et al.*, 2005; Chen *et al.*, 2007). The conditions and procedures for probe labelling, hybridization, post-hybridization washing and immunological detection of the probe with a disodium 3-[4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1]decan}-4-yl]phenyl phosphate (CSPD) chemiluminescent substrate for alkaline phosphatase were carried out according to the manufacturer's instructions (Roche).

Pathogenicity assay. Assay for fungal pathogenicity was carried out on detached tobacco leaves (*Nicotiana tabacum* 'Burley 21') with conidia suspensions (5×10^4 conidia ml^{-1}) as described previously (Choquer *et al.*, 2005).

RESULTS

Characterization of the *CTB5*, *CTB6* and *CTB7* genes

In total, eight *CTB* genes (*CTB1–8*) are closely linked in the genome of *C. nicotianae* and probably involved in biosynthesis of cercosporin (Fig. 1a). In this study we utilized a genetic approach to further define the roles of *CTB5*, *CTB6* and *CTB7* in cercosporin biosynthesis. Both *CTB5* and *CTB7* are located near the left border of the cercosporin biosynthetic gene cluster (Fig. 1b). The coding regions of *CTB5* and *CTB7* are separated by 867 bp and are transcribed in the same direction. The *CTB5* ORF contains 1380 bp with no introns and is predicted to encode a polypeptide of 459 aa. The translation product of *CTB5* displays 30–59 % identity and 50–72 % similarity to numerous uncharacterized, conserved, hypothetical proteins from sequenced genomes of fungi (data not shown). The amino acid sequence deduced from the *CTB5* ORF also resembles various oxygen- and FAD/FMN-dependent oxidoreductases of various bacteria in the databases. CTB5 has a putative TonB-dependent receptor protein signature, a potential oxygen-interacting site, a FAD-binding site, a tyrosine sulfation site, and a putative NADH-binding site (Fig. 2).

The *CTB6* gene is located near the right border of the *CTB* gene cluster (Fig. 1b), consists of 1074 bp with no introns, and presumably encodes a 357 aa polypeptide. The translation product of *CTB6* has 26–40 % identity and 48–61 % similarity to numerous NADPH-dependent reductases, oxidoreductases or dehydrogenases of various micro-organisms. An alcohol dehydrogenase family signature and a motif that probably interacts with NADPH were identified in CTB6 (Fig. 2).

The *CTB7* ORF consists of 1401 bp interrupted by a single intron of 48 bp and is predicted to encode a protein of 450 aa. The translation product of *CTB7* has 22–33 %

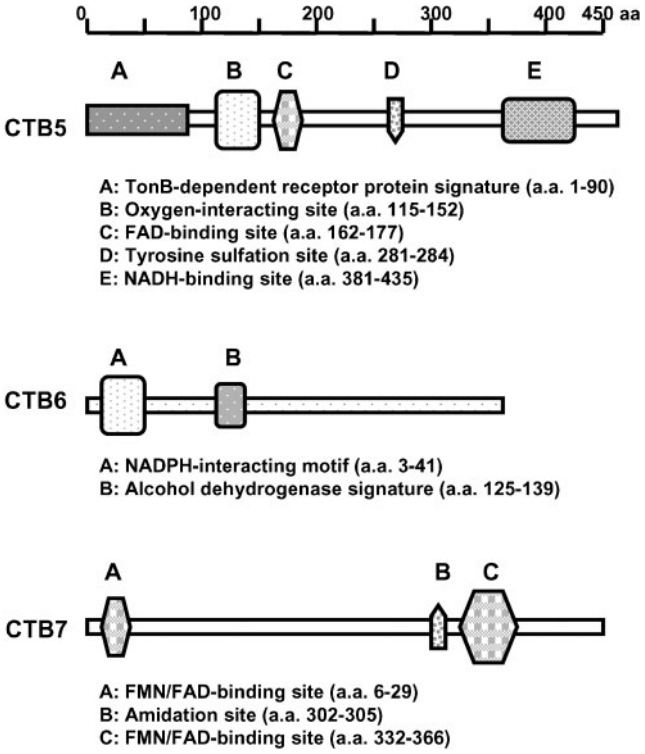

Fig. 2. Diagrams of the CTB5, CTB6, and CTB7 proteins, showing unique motifs and various consensus sequences.

identity and 38–51 % similarity to a wide variety of FAD/FMN-dependent oxidoreductases, hydrolases, or monooxygenases of fungi and bacteria. CTB7 has two FMN/FAD or flavin-containing monooxygenase-binding sites and an amidation site (Fig. 2).

Disruption of the *CTB5*, *CTB6* and *CTB7* genes

Targeted gene disruption was performed to evaluate the functions of *CTB5*, *CTB6* and *CTB7* associated with cercosporin production in *C. nicotianae*. Transformants were screened for cercosporin production on a thin PDA plate. In total, 53 of 202 (26 %) transformants recovered from *CTB5* disruption, 10 of 141 (7.1 %) from *CTB6* disruption, and 3 of 16 (19 %) from *CTB7* disruption were completely defective in cercosporin accumulation.

Successful disruption of each *CTB* locus was validated by Southern blot analysis. Hybridization of *Eco*RI/*Hin*dIII-digested genomic DNA from the wild-type and six putative *ctb5* disruptants to a *CTB5* gene probe identified a 3.0 kb band in wild-type (Fig. 3a, b). In contrast, all transformants had a 2.4 kb hybridizing band instead, due to the insertion of *HYG* within *CTB5* and the presence of an additional *Eco*RI site in the *HYG* gene cassette. Three hybridizing bands, >4 kb (indicated by arrowheads in Fig. 3b), in DNA prepared from the *ctb*5-D1 and D9 disruptants, were due to the ectopic integrations of split marker fragments in

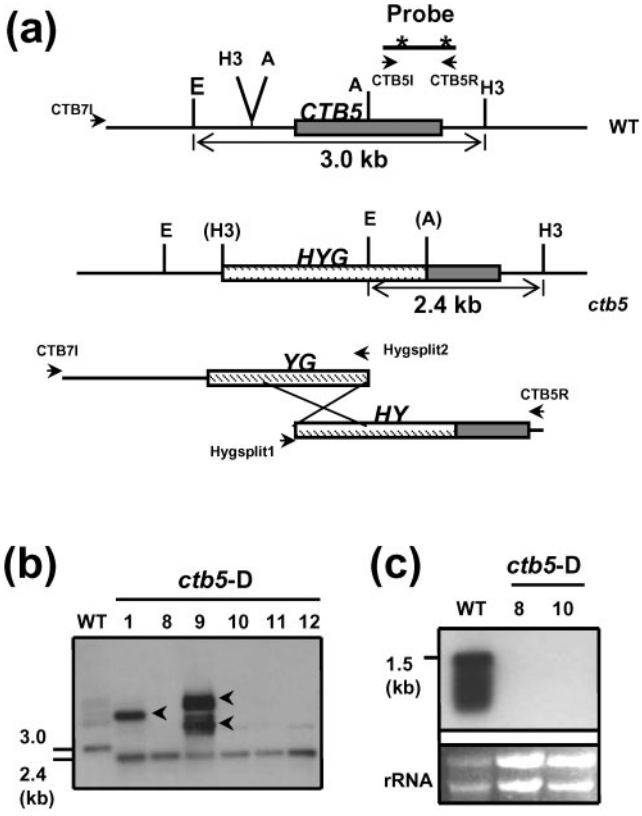

Fig. 3. Targeted gene disruption of *CTB5* in *C. nicotianae*. (a) Schematic depiction of restriction maps of *CTB5* in wild-type (WT) and *ctb5*-disrupted fungal genomes. The split-hygromycin phosphotransferase B gene (*HYG*) marker fused with the truncated *CTB5* fragments was amplified and used for disruption. Oligonucleotide primers (ctb7I, ctb5R, ctb51, hygsplit 1 and hygsplit 2) used for cloning, probe labelling and amplification of the split markers are also indicated. Restriction enzyme site abbreviations: A, *Age*I; E, *Eco*RI; H3, *Hin*dIII. Note: drawing is not to scale. (b) Southern blot hybridization of genomic DNA from the wild-type and six *ctb5* knockouts confirms gene-specific replacement at the *CTB5* locus. Fungal DNA was digested with *Eco*RI and *Hin*dIII, electrophoresed, blotted onto a nylon membrane, and hybridized to a *CTB5*-specific probe as indicated above. The hybridizing bands indicated by arrows in DNA prepared from two disruptants (*ctb5*-D1 and D9) may be due to the ectopic integrations in the genome. (c) Northern blot analysis of total RNA isolated from the wild-type and two Δ*ctb5* mutants (D8 and D10). Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

the genome. Northern blot analysis of total RNA from two *ctb5* disruptants also confirmed the null mutation at *CTB5* (Fig. 3c).

As shown in Fig. 4(a, b), hybridization of *Nco*I/*Xho*I-digested genomic DNA to a *CTB6* gene probe yielded a 2.0 kb hybridizing band in wild-type and a 1.8 kb band in three putative Δ*ctb6* disruptants due to the presence of an extra *Nco*I site in the inserted *HYG* fragment. The hybridization patterns confirmed a successful disruption

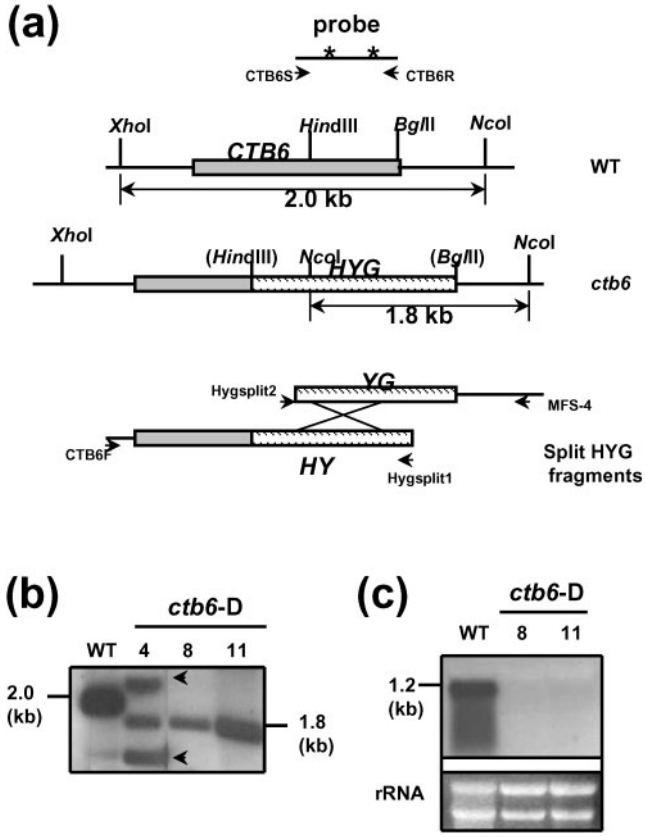

Fig. 4. Targeted gene disruption of *CTB6* in *C. nicotianae*. (a) Restriction maps of *CTB6* in wild-type (WT) and Δ*ctb6*-disrupted fungal genomes, and the split-hygromycin phosphotransferase B gene (*HYG*) marker fused with the truncated *CTB6* fragments. Oligonucleotide primers used for cloning, probe labelling and amplification of the split markers are also indicated. (b) Southern blot analysis of genomic DNA prepared from the wild-type and three *ctb6* knockouts. Fungal DNA was digested with *Nco*I and *Xho*I, electrophoresed, blotted onto a nylon membrane, and hybridized to a *CTB6*-specific probe. The hybridizing bands indicated by arrows in DNA prepared from *ctb6*-D4 were probably due to the ectopic integration of genomic DNA. (c) Northern blot analysis of total RNA isolated from the wild-type and two Δ*ctb6* mutants (D8 and D11). Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

at the *CTB6* locus. Hybridizing bands (>2.0 kb and <1.5 kb) due to the ectopic integration of PCR fragments were detected in DNA prepared from the *ctb6*-D4 disruptant. Northern blot hybridization of total RNA from two *ctb6* disruptants further validated the null mutation at *CTB6* (Fig. 4c).

The hybridization profiles shown in Fig. 5(a, b) also confirmed targeted disruption of *CTB7*. The *CTB7* probe hybridized to 2.7 and 3.4 kb *Bcl*I fragments in DNA purified from the wild-type and five putative *ctb7* disruptants, respectively. Ectopic insertions were also detected in DNA from the Δ*ctb7*-D1, D4 and D7 disruptants. Hybridization of total RNA from wild-type and two *ctb7* disruptants to a *CTB7* probe also confirmed the null mutation at *CTB7* (Fig. 5c).

Functional complementation

To further evaluate the roles of *CTB5*, *CTB6* and *CTB7* in relation to cercosporin biosynthesis, genetic complementation was carried out by co-transforming a functional *CTB5*, *CTB6* or *CTB7* gene cassette (under its own promoter) with plasmid pCB1532 into the respective disruptants (*ctb5*-D8, *ctb6*-D18 and *ctb7*-D2). Transformants were selected on media containing sulfonylurea then tested for cercosporin production on thin PDA plates. The results

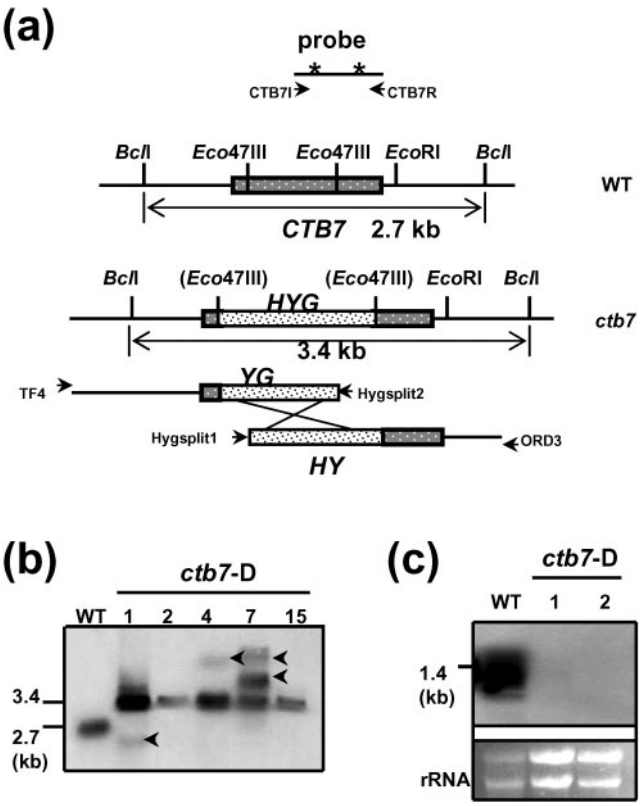

Fig. 5. Gene replacement of *CTB7* in *C. nicotianae*. (a) Restriction maps of *CTB7* in wild-type (WT) and Δ*ctb7*-disrupted fungal genomes, and the split-hygromycin phosphotransferase B gene (*HYG*) marker fused with the truncated *CTB7* fragments. Oligonucleotide primers used for cloning, probe labelling and amplification of the split markers are also indicated. (b) Southern blot analysis of genomic DNA prepared from the wild-type and five putative *ctb7* disruptants. Fungal DNA was digested with *Bcl*I, electrophoresed, blotted onto a nylon membrane, and hybridized to a *CTB7*-specific probe. The hybridizing bands indicated by arrows in DNA prepared from three disruptants (*ctb6*-D1, D4 and D7) were due to the ectopic integration of genomic DNA. (c) Northern blot analysis of total RNA isolated from the wild-type and two Δ*ctb7* mutants (D1 and D2). Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

indicated that transformation of full-length *CTB5*, *CTB6* or *CTB7* genes into the *ctb5*-D8, *ctb6*-D18 or *ctb7*-D2 disruptants, respectively, enabled the mutants to restore cercosporin production to levels comparable to the wild-type (see below).

Cercosporin production

The amounts of cercosporin in KOH extracts were determined, revealing that the absorbance values obtained from the *ctb5*, *ctb6* or *ctb7* disruptants were indistinguishable from the controls (agar plugs only) and were considered to be zero (Table 1). To determine if a trace amount of cercosporin was produced by the disrupted mutants, cercosporin was extracted by ethyl acetate from agar plugs with fungal mycelia and the extracts were analysed by TLC. As shown in Fig. 6, the wild-type and the *CTB5*-, *CTB6*- and *CTB7*-complemented strains produced a red pigment (cercosporin), whereas the *ctb5*, *ctb6* or *ctb7* disruptants produced no detectable cercosporin. Thus, disruption of the *CTB5*, *CTB6* or *CTB7* gene completely blocked cercosporin biosynthesis, but disruptants accumulated a yellowish or purplish pigment that was barely visible on fluorescent-TLC plates (Fig. 6).

Transcriptional inhibition of the *CTB* genes in the *ctb5*-, *ctb6*- and *ctb7*-disrupted mutants

Northern blot analysis was performed to determine if disruption of the *CTB5*, *CTB6* or *CTB7* gene would affect expression of the other *CTB* genes in the cluster (Fig. 7). The results indicated that expression of *CTB5* was almost undetectable in the *ctb6* and *ctb7* null mutants (Fig. 7a). Accumulation of the *CTB6* transcript was slightly reduced in two *ctb5* null mutants, but completely abolished in two *ctb7* null mutants (Fig. 7b). Expression of *CTB7* was barely detectable in the *ctb5* and *ctb6* null mutants (Fig. 7c). Similarly, expression of *CTB1* and *CTB8* was drastically down-regulated in the *ctb5* and *ctb6* null mutants, and accumulation of the *CTB1* but not the *CTB8* gene transcript was also reduced in two *ctb7* null mutants (Fig. 8).

Sensitivity to cercosporin and other singlet-oxygen-generating photosensitizers

To determine if *CTB5*, *CTB6* or *CTB7* play a role in cellular resistance to cercosporin and other singlet-oxygen-generating photosensitizers, wild-type and *ctb5*, *ctb6* and *ctb7* disruptants were grown on media containing exogenous photosensitizing compounds (cercosporin, eosin Y, haematoporphyrin, methylene blue or toluidine blue). As compared to the wild-type, *ctb5*, *ctb6* or *ctb7* null mutants did not show significant growth retardation in the presence of exogenous cercosporin or other photosensitizers (Table 1). For comparison purpose, strain 205C3, which is deficient in a zinc finger transcriptional factor, CRG1, required for normal cercosporin production and resistance (Chung *et al.*, 2003a), was tested. In agreement with the previous findings (Chung *et al.*, 2003a), the 205C3 mutant

Table 1. Accumulation of cercosporin and radial growth of *C. nicotianae* isolates in the presence of singlet-oxygen-generating photosensitizers

Isolate*	Cercosporin accumulation (nmol)†	Radial growth (mm)‡				
		CR	EY	HP	MB	TB
		10 μM	10 μM	10 μM	100 μM	100 μM
WT	107.4±12.7	8.6±0.8	10.9±0.7	10.4±0.1	9.5±1.0	10.7±1.2
ctb5-D8	0	9.5±1.5	14.2±0.2	12.6±1.3	11.3±0.3	10.4±0.2
ctb5-D11	0	9.8±1.6	13.2±1.4	13.0±1.5	13.0±1.5	12.8±0.8
CTB5C1	121.6±42.3	8.6±1.4	12.5±1.0	12.4±0.9	9.5±0.5	9.9±0.2
ctb6-D8	0	10.1±1.9	12.3±0.1	11.5±0.3	10.7±0.5	11.2±0.7
ctb6-D11	0	9.8±0.6	11.8±0.5	12.0±0.7	11.1±0.9	11.5±0.8
CTB6C5	113.1±15.4	9.6±0.8	12.2±0.7	12.2±0.4	11.5±0.8	10.5±0.3
ctb7-D2	0	9.7±0.9	14.0±0.3	12.1±0.8	12.8±0.6	12.2±0.4
ctb7-D4	0	9.7±1.9	12.1±0.4	11.2±0.4	10.1±1.3	10.0±0.5
CTB7C2	119.6±10.4	9.1±1.3	11.9±0.9	10.9±0.7	10.9±1.6	10.9±0.7
205C3	45.5	5.1	11.6	12.0	12.2	11.4

*Fungal isolates: WT, wild-type; *ctb*-D, *ctb5*, *ctb6* and *ctb7*, disruptants; CTB5C1, CTB6C5 and CTB7C2, complementation strains; 205C3, strain deficient in zinc finger transcription factor, CRG1 (Chung *et al.*, 2003a).

†Fungal isolates were grown on thin PDA plates for 5 days. Cercosporin was extracted with 5 M KOH and quantified by measuring A_{480}. The blank control contained agar plugs only. Experiments were repeated twice except for 205C3. Data are the mean±SE of two experiments.

‡Fungal isolates were grown on complete medium containing exogenous cercosporin, eosin Y, haematoporphyrin, methylene blue, or toluidine blue under constant fluorescent light at room temperature; radial growth was determined after 7 days. Values are the mean±SE of three experiments with three replicates for each treatment.

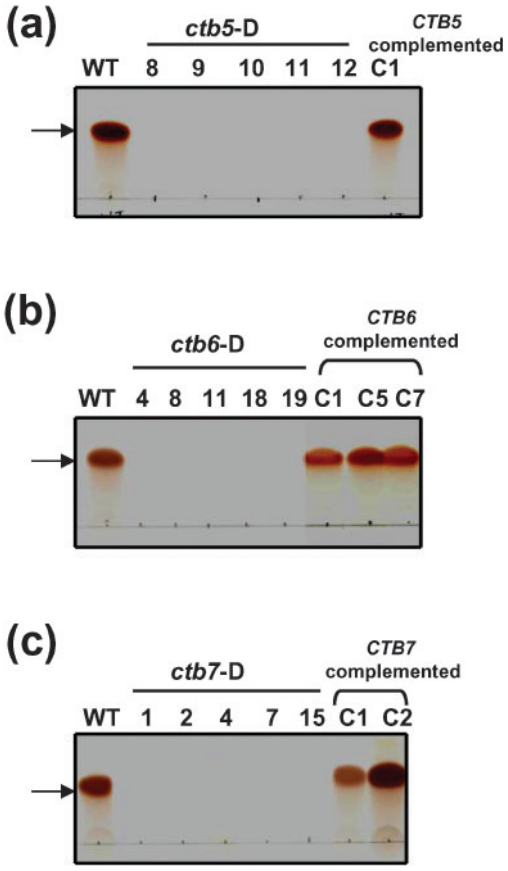

Fig. 6. TLC analysis of cercosporin produced by wild-type (WT), *ctb5*, *ctb6*, and *ctb7* disruptants and complemented strains of *C. nicotianae*. Fungal isolates were grown on PDA under continuous light for 5 days and cercosporin was extracted with ethyl acetate.

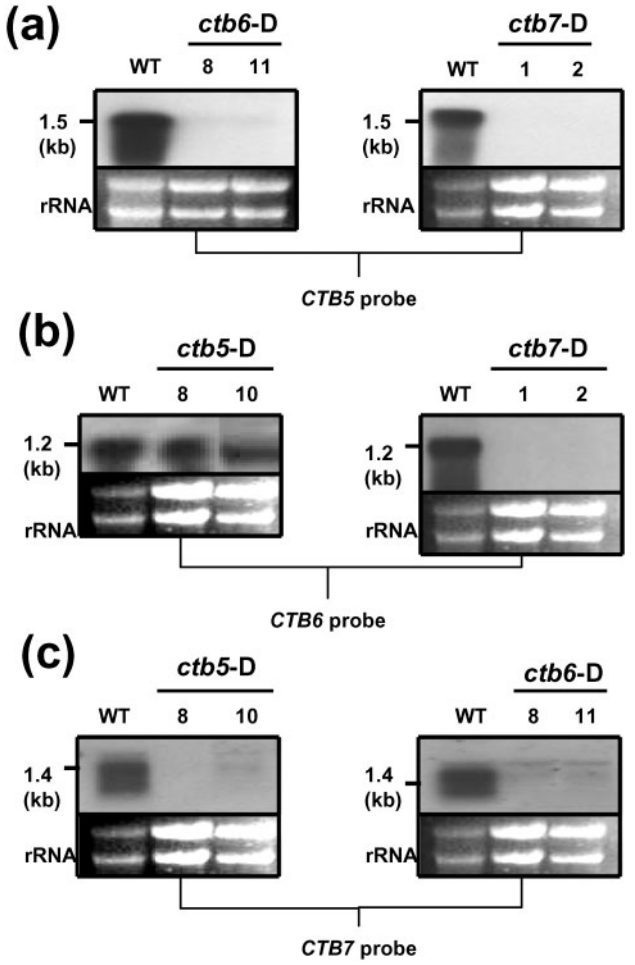

Fig. 7. Northern blot analysis of total RNA prepared from the wild-type (WT), and the *ctb5* (D8 and D10), *ctb6* (D8 and D11) and *ctb7* (D1 and D2) disruptants of *C. nicotianae*. (a) Accumulation of *CTB5* in *ctb6* and *ctb7* disruptants. (b) Expression of *CTB6* in *ctb5* and *ctb7* disruptants. (c) Expression of *CTB7* in *ctb5* and *ctb6* disruptants. Total RNA was electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to probes at 65 °C as indicated. Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

exhibited partial sensitivity to cercosporin but not to other photosensitizers, and produced less than 50 % of the cercosporin produced by the wild-type (Table 1).

Pathogenicity

As assayed on detached tobacco leaves, the *ctb5*, *ctb6* or *ctb7* disruptants caused fewer lesions compared to the wild-type. However, the complementation strains incited necrotic lesions on tobacco, indistinguishable from those induced by the wild-type (data not shown).

DISCUSSION

Cercosporin is a polyketide compound. Biosynthesis of cercosporin has been predicted to start with decarboxylation of acetyl-CoA and malonyl-CoA units to synthesize petaketide, followed by ring closure, oxidation, hydration and methylation to form the polyketomethylene backbone of cercosporin (Okubo *et al.*, 1975). Chromosome walking coupled with sequence analysis led to identification of the cercosporin toxin biosynthesis (*CTB*) cluster. The core *CTB* gene cluster in *C. nicotianae* consists of a transcriptional regulator gene, a potential transporter gene, and six biosynthetic genes (Chen *et al.*, 2007). To fully determine the function of the *CTB1–8* genes associated with cercosporin production, we performed genetic and molecular analysis of *C. nicotianae* strains with loss- and gain-of-function mutations in each *CTB* gene (Chen *et al.*, 2007; Choquer *et al.*, 2005, 2007; Dekkers *et al.*, 2007). In this present study we analysed three putative oxidoreductase-encoding genes (*CTB5*, *CTB6* and *CTB7*) that were localized in the *CTB* cluster, and obtained experimental evidence to support their crucial roles in cercosporin biosynthesis.

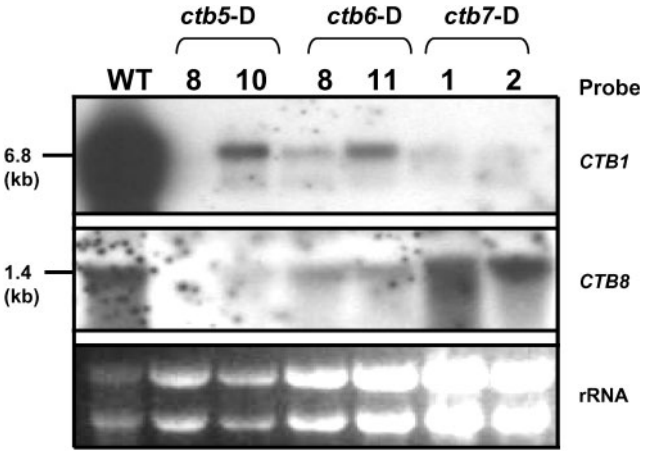

Fig. 8. Northern blot analysis of total RNA prepared from the wild-type (WT), and the *ctb5* (D8 and D10), *ctb6* (D8 and D11) and *ctb7* (D1 and D2) disruptants of *C. nicotianae*, indicative of a 'feedback' transcriptional inhibition of the *CTB1* and *CTB8* genes. Total RNA was electrophoresed in formaldehyde-containing gels, blotted onto nylon membranes and hybridized to a *CTB1* or *CTB8* probe as indicated. Ethidium-bromide-stained rRNA is shown to indicate the relative loading of the samples.

Although the biochemical function of CTB5, CTB6 and CTB7 as oxidoreductases remains to be proven, molecular and genetic analyses clearly indicated that they were required for cercosporin biosynthesis. A prior study revealed that accumulation of *CTB5*, *CTB6* and *CTB7* transcripts was co-ordinately controlled by the CTB8 transcriptional activator (Chen *et al.*, 2007). In the present study, disruption of *CTB5*, *CTB6* or *CTB7* yielded mutants that were completely impaired in cercosporin production, yet retained the wild-type level of resistance to cercosporin and other singlet-oxygen-generating photosensitizers. We have also demonstrated that disruption of each of the *CTB5*, *CTB6* or *CTB7* genes markedly reduces transcriptional accumulation of the other *CTB* gene transcripts, consistent with the previous finding of the presence of a feedback inhibition mechanism (Chen *et al.*, 2007). However, it appears that such inhibition was not completely stringent since expression of *CTB6* was slightly reduced in two *ctb5* null mutants, but completely undetectable in the *ctb7* null mutants (Fig. 7). Furthermore, expression of *CTB8* was apparently down-regulated in the *ctb5* or *ctb6* null mutants, but was normal in the *ctb7* mutants (Fig. 8).

CTB5 has amino acid similarity to many oxygen- and FAD/FMN-dependent oxidoreductases of bacteria and fungi, including vanillyl-alcohol oxidases, D-lactate dehydrogenases, 6-hydroxy-D-nicotine oxidases (accession nos. P56216, P06149, ZP_00522304, and CAA29416), and mitomycin radical oxidases of *Streptomyces* (accession no. P43485). These enzymes catalyse the oxidation of a wide variety of substrates for energy production and conversion (Rule *et al.*, 1985; van den Heuvel *et al.*, 2000). The mitomycin radical oxidase, however, oxidizes the reduced form of mitomycins and is involved in cellular self-defence against mitomycin in *Streptomyces lavendulae* (August *et al.*, 1994). The enzyme encoded by *CTB5* is proposed to utilize FAD and/or FMN as a cofactor and catalyse the oxidation steps in the cercosporin biosynthetic pathway. CTB5 has a putative TonB-dependent receptor signature. The TonB protein is involved in the passive uptake of large and low-affinity substrates by interacting with outer-membrane receptors in *Escherichia coli* (Bell *et al.*, 1990). Without TonB, the receptors bind their substrates but fail to transport them into the periplasmic space. In addition, CTB5 also has a tyrosine sulfation site that is physiologically associated only with proteins or domains that are transported or reside in the Golgi lumen (Huttner, 1988). It will be interesting to determine how those domains contribute to CTB5 function.

The protein encoded by *CTB6* displays considerable similarity to numerous NADPH-dependent reductases, oxidoreductases or dehydrogenases in bacteria, yeasts and plants. Some members include aldehyde reductases, nucleoside-diphosphate sugar epimerases, carbonyl reductases, dihydroflavonol 4-reductases and cinnamyl-alcohol dehydrogenases (accession nos. 1Y1PA, XP_71038, ZP_00592614, and ZP_00528210). A computer search identified an alcohol dehydrogenase (adh) family signature and a NADPH-binding motif in CTB6. A CTB6 homologue in *Saccharomyces cerevisiae* (accession no. NP_011476) was thought to catalyse NADPH-dependent reduction of the bicyclic diketone bicyclo[2,2,2]octane-2,6-dione to the chiral ketoalcohol-6-hydroxybicyclo[2,2,2]octane-2-one (Goffeau *et al.*, 1996). It is tempting to speculate that the CTB6 enzyme catalyses an NADPH-dependent reduction or hydration step during ring closure of pentaketide in the cercosporin biosynthetic pathway.

The translation product of *CTB7* has an amidation site and two FMN/FAD or flavin-containing monooxygenase-binding sites. Proteins having similarity to the CTB7 include flavoprotein monooxygenases and pyridine nucleotide-disulfide oxidoreductases of *Pseudomonas* (accession nos. YP_2333205 and YP_272409), and FAD-dependent oxidoreductases of *Xanthomonas* (accession nos. YP_363434 and AAM36534). CTB7 also has similarity to a *Xanthomonas campestris* pv. *zinniae* oxidoreductase (accession no. AAY86766) which has recently been shown to be involved in cercosporin degradation (Taylor *et al.*, 2006). We propose that the function of CTB7 in the cercosporin biosynthetic pathway is probably to catalyse a hydration or reduction step during ring closure to form the polyketomethylene skeleton of cercosporin prior to the methylation steps.

Biosynthetic gene clusters often include one or more genes for cellular self-protection in some fungi. For example, TRI12 is an efflux pump that is involved in self-protection against trichothecene in *Fusarium sporotrichioides*

(Alexander *et al.*, 1999), and TOXA is a putative HC-toxin transporter in *Cochliobolus carbonum* (Pitkin *et al.*, 1996). Cercosporin is toxic to many cells due to the production of singlet oxygen; however, *Cercospora* species are very resistant to cercosporin (Daub *et al.*, 2005). The mechanisms involved in cercosporin resistance have been attributed to the ability of *Cercospora* species to transiently reduce cercosporin, perhaps via membrane reductases (Daub *et al.*, 1992, 2000; Leisman & Daub, 1992; Sollod *et al.*, 1992). Recently, expression of a yeast FAD-dependent reductase in tobacco conferred resistance to cercosporin (Panagiotis *et al.*, 2007). Despite the fact that the *CTB5*, *CTB6* and *CTB7* genes encode putative oxidoreductases, targeted gene disruption indicated that none of them is responsible for cercosporin self-resistance. Loss of the ability to synthesize cercosporin was the only phenotypic change caused by mutation of the *CTB5*, *CTB6* or *CTB7* genes. Conidiation of *ctb5*, *ctb6* and *ctb7* disruptants was not affected (data not shown). Finally, the *ctb5*, *ctb6* and *ctb7* disruptants produced fewer lesions compared to the wild-type on tobacco leaves, consistent with previous findings that cercosporin is an important virulence factor (Chen *et al.*, 2007; Choquer *et al.*, 2005, 2007; Dekkers *et al.*, 2007).

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